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14. ABSTRACT

The main goals of this proposal are: 1) to develop and optimize a rapid method to assess the ability of breast tumor cell lines to repair specifically DNA interstrand crosslinks (ICLs). 2) to apply this technology to a set of human breast cancer cell lines. In addition, we propose to compare ICLs repair with surrogate markers for ICL repair. The predicted outcome of these studies is to identify a subset of human breast tumor cell lines that are cisplatin-sensitive and to correlate this sensitivity with their ability to repair ICLs. We hypothesized that breast cancer cell lines with defects in ICL repair will fall into the basal-like/triple-negative breast cancer category of tumor cell lines and correlate with an increased sensitivity to cisplatin.

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INTRODUCTION

Recent advances in the treatment of breast cancer based on the measurement of ER/PR and HER2 in tumors have significantly facilitated the way in which a treatment regime is selected for breast cancer patients, and vastly improved their prognosis. These advances have been possible because a better understanding of the breast cancer subtypes has been acquired and reliable biomarkers have been developed.

In contrasts to the abovementioned tumors, no specific treatment or “personalized medicine” is currently available to treat tumors that are negative for ER/PR and HER2 markers: triple negative breast cancers, TNBCs. Indeed, patients who suffer from these tumors have a poor prognosis despite response to chemotherapy in the neoadjuvant setting, possibly because treatment relies primarily on random selection of a chemotherapy regimen (Metzger-Filho et al., 2012).

In this proposal we started to address the vital need to develop prognostic markers that will guide rational therapeutic strategies for this subset of tumors. Specifically, we proposed to test the hypothesis that the ability to repair DNA interstrand crosslinks (ICLs) correlates with resistance to crosslinking therapy (nitrogen mustards, platinum drugs) in TNBCs.
Our long-term is to identify prognostic markers to predict the sensitivity or resistance of TNBCs to crosslinking agents.

BODY

Progress was made for the three original tasks of the proposal. However, some unanticipated technical problems prevented us to complete the studies as originally outlined in the statement of work (see below).

Task 1a. We have optimized transfection conditions for a subset of breast tumor cell lines from the ATCC. We performed optimization on cell lines from TNBC cells that were already available at our institute. These lines have been previously profiled for their mRNA expression, histological or pathological subtype and for a fraction of them, their genetic and genomics characteristics (Metzger-Filho et al., 2012). Notably, these cell lines recapitulate critical aspects of the disease and reflects its heterogeneity. Some are ER positive and are estrogen-dependent for growth, whereas others are HER2 positive and depend on HER2 activation. Within the entire cell line panel, 24 of the cell lines have TNBCs characteristics.

We tested several conditions, including traditional transfection methods, lipofectamine-based methods, including improved version of that technique, nanojuice® and nucleofection. We observed that all cell lines transfected with very variable efficiency using different protocols. While nanojuice® worked best for some cell lines, it was not the optimal technique for others, which prompted us to modify our plan of attack to compare ICL repair abilities between cell lines (see below).

Task 1b. We generated the ICL containing plasmids in which the ICL is chemically generated within a double-strand oligonucleotide prior to being ligated in a GFP-containing vector (Ben-Yehoyada et al., 2009; Williams et al., 2012). To generate the repair template, a ds-oligonucleotide harboring a single ICL mimicking a platinum crosslink is chemically synthesized. The ds-oligonucleotide is ligated into the multiple cloning sites (MCS) of a pIRES2-AcGFP1 vector. pIRES2-AcGFP1 contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus (ECMV) between the MCS and the *Aequorea coerulescens* green fluorescent protein (AcGFP1) coding region. This permits both the sequence containing the ICL (cloned into the MCS) and the AcGFP1 gene to be transcribed into a single bicistronic mRNA *if the ICL is repaired*. pIRES2-AcGFP1 is designed for the efficient selection (by FACS-Fluorescent activated cell sorting) of transiently transfected mammalian cells expressing AcGFP1 (Figure 1). The multiple cloning site and the GFP sequence are separated by several hundreds of nucleotides in the pIRES2-AcGFP1 vector. We observed that this configuration resulted in some background fluorescence. Therefore, we primarily used a second vector in

which the ICL oligo (with and ATG in frame) is ligated immediately upstream of the AcGFP1 start codon (pAcGFP1-N3). If repaired accurately, a new ATG is generated within the repaired ICL and translation of AcGFP1 with a 6 amino acids N-terminal tag takes place. Since ICLs covalently link the two strands of DNA, they block transcription. Therefore, expression of GFP

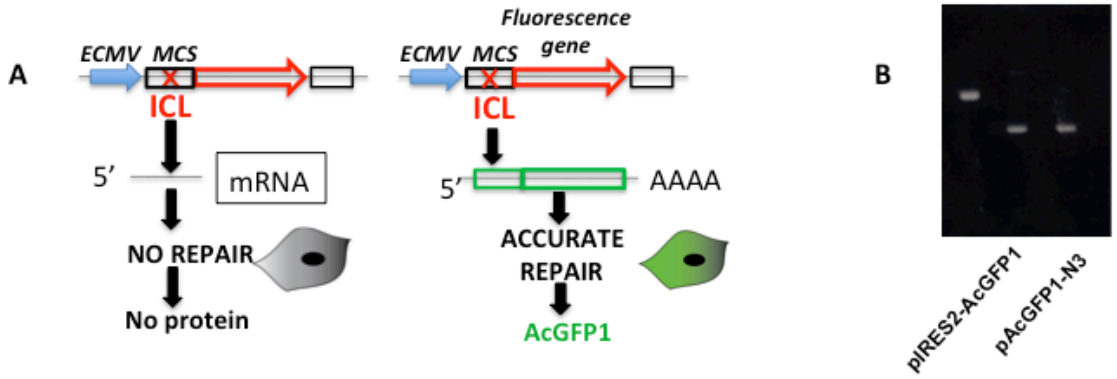

Figure 1: Design and purification of the GFP-ICL vectors. A. Experimental scheme. The ICL is located immediately 5' to an ATG. GFP mRNA and protein are expressed following accurate repair; cells expressing GFP are counted by FACS as a measure of repair. B. Purifications of the two ICL-containing plasmid following ligation of the ICL-containing oligonucleotide and purification by CsCl centrifugation.

indicates that the ICL lesion has been repaired. The plasmids were subsequently tested in a variety of cells: mouse embryonic fibroblasts as well as human HeLa and U2OS cells to test the plasmid and to optimized conditions for sorting cells expressing GFP by FACS (Figure 1).

Task 1c. We optimized conditions for FANCD2 under conditions or DNA replication stress as well as following treatment with DNA crosslinking agents. An example of FANCD2 immunostaining is presented in Figure 2.

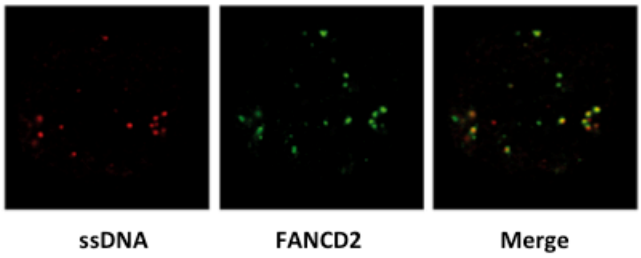

Figure 2: Cells were incubated for 12 h with hydroxyurea, followed by the crosslinking drug. ssDNA foci was stained with red fluorescence (left panel, FANCD2 was revealed with green fluorescence (middle panel) yellow foci show co-localization (right panel).

Task 2. ICL repair of the ICL-GFP plasmid is sensitive to the cellular concentration of transfected plasmid. Therefore, it is important to establish conditions with similar transfection efficiencies. We made a lot of efforts to obtain such conditions across the panel of breast tumor cell lines. However, we were not able to reproducibly obtain conditions yielding similar transfections levels in the subset of cell lines we tested.

Therefore, we initiated another strategy. Since our ultimate goal is to characterize the ICL repair capability of TNBCs, we thought to directly compare the ability of cells (from the breast cancer panel) to repair ICLs and to perform homology-directed repair (HDR). The rationale being that the majority of TNBCs show a defect in HDR (such as the defect observed in BRCA1-deficient cells). In this setting, instead of measuring the absolute ability of a cell line to repair ICLs, we now measure the relative ability to repair ICL/ability to perform HDR. This value is independent of the transfection efficiency of a given cell line. We are currently optimizing this approach with encouraging preliminary results. Unfortunately, these efforts are starting to pay off beyond the time frame of the DOD award.

While we were optimizing the ICL repair assay and transfection conditions on different cell lines, we applied it to isogenic cells proficient or deficient for the translesion synthesis polymerase Pol Kappa. We found that DNA polK deficient cells show a significant defect in their ability to repair a single ICL. These critical experiments further validated the use of the ICL-GFP reporter assay and have been recently published (Williams et al., 2012).

Task 3. One of the goals of the proposal was to correlate the ability of TNBCs cells to repair a single ICL with their sensitivity to crosslinking drugs such as cisplatin as originally described in task 3b. Because of the technical difficulties we encountered obtaining transfection conditions with similar efficiency across the cell lines (see above), we were not able to assess this correlation in a controlled and satisfactory fashion. Because this technical issue arose probably

because of high genetic heterogeneity, we sought to provide a proof of principle of this concept using the isogenic cells proficient and deficient for PolK. Therefore, as a proof of principle of the rationale, we did correlate the ability of PolK-deficient cells with their sensitivity to two crosslinking drugs: cisplatin and MMC. We found that the sensitivity of PolK-deficient cells to these drugs is higher than the sensitivity of WT cells (Figure 3). Therefore, we conclude that our ICL-GFP assay is a good prognostic marker for the sensitivity of cells to crosslinking drugs.

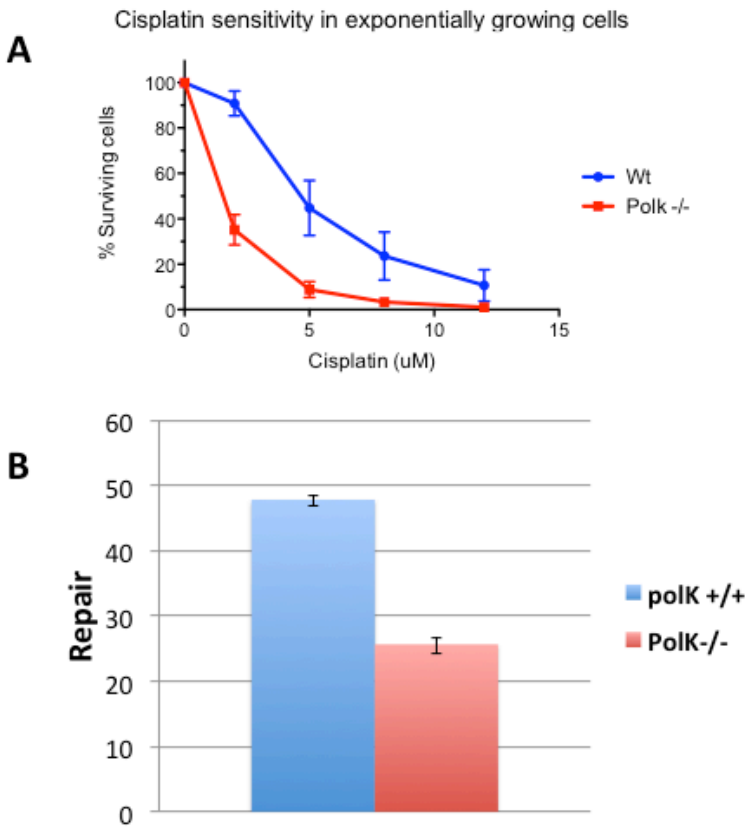

Figure 3: Cisplatin sensitivity of PolK-deficient cells correlates with their ICL repair capacities. A. Clonogenic survival assay of PolK-deficient and proficient (WT) cells following cisplatin treatment. B. ICL repair efficiency of Polk-proficient (blue) and –deficient (red) cells monitored using the assay described in Figure 1.

KEY RESEARCH ACCOMPLISHMENTS

- Developed a unique reporter assay for ICL repair that is functional in replicating cells as well as in G1 arrested cells.
- Demonstrated that the ability to repair a single ICL inversely correlates with the cellular sensitivity to crosslinking drugs.

REPORTABLE OUTCOME

Williams, H.L., Gottesman, M.E., and Gautier, J. (2012). Replication-independent repair of DNA interstrand crosslinks. Mol Cell *47*, 140-147.

The personnel involved in this research project:

- Dr. Jean Gautier
- Dr. Hannah Williams

CONCLUSION

Despite technical difficulties probably due to the genetic heterogeneity of breast tumor cells in culture, our experiments have allowed us to establish a direct correlation between the resistance to crosslinking drugs used for breast cancer therapy, such as cisplatin, with the cell's ability to repair ICLs. This is a significant advance in the field since crosslinking agents generate a variety of aberrant DNA structures of which ICLs are only minority.

In turn, this will allow using our repair assay based on the use of a ICL-GFP reporter as a tool to evaluate the sensitivity of cells to crosslinking therapy. Therefore, we defined a potential new prognosis marker for breast cancer therapy. Notably, the subtype of breast tumors which are currently treated with traditional chemotherapies are the TNBCs, the class of tumors with the worst prognosis.

REFERENCES

Ben-Yehoyada, M., Wang, L.C., Kozekov, I.D., Rizzo, C.J., Gottesman, M.E., and Gautier, J. (2009). Checkpoint signaling from a single DNA interstrand crosslink. Mol Cell *35*, 704-715.

Metzger-Filho, O., Tutt, A., de Azambuja, E., Saini, K.S., Viale, G., Loi, S., Bradbury, I., Bliss, J.M., Azim, H.A., Jr., Ellis, P.*, et al.* (2012). Dissecting the heterogeneity of triple-negative breast cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology *30*, 1879-1887.

Williams, H.L., Gottesman, M.E., and Gautier, J. (2012). Replication-independent repair of DNA interstrand crosslinks. Mol Cell *47*, 140-147.